DESCRIPTION

PROTEIN HAVING PESTICIDAL ACTIVITY, DNA ENCODING THE PROTEIN, AND NOXIOUS ORGANISM-CONTROLLING AGENT AND METHOD

This application is a 371 of PCT/JP01/06660, filed August 2, 2001, which claims priority to JP 2000-236140 filed August 3, 2000.

TECHNICAL FIELD

The present invention relates to a protein having a pesticidal activity, DNA encoding the protein, a noxious organism-controlling agent and -controlling method as well as to a novel *Bacillus thuringiensis serovar galleriae* SDS502 strain (hereinafter, sometimes abbreviated as SDS502).

BACKGROUND ART

Bacillus thuringiensis (hereinafter, sometimes abbreviated as Bt) forms endospores like other *Bacillus* bacteria. The spores germinate and grow into vegetative cells in the presence of suitable nutritional components. The vegetative cells repeat cell division successively and sooner or later turn into sporangia that form endospores and crystal protein in the cells due to exhaustion of nutritional components, environmental changes, and so forth. Further, the cells are destructed to release the endospores and crystal protein.

Insects eat the spores and crystal protein Bt produces. When they reach the mesenteron in the digestive tract, the protein is dissolved under strongly alkaline conditions of the digestive juice to produce a protoxin, which then is converted by a proteolytic enzyme into an active ingredient (toxin). The active ingredient binds to a receptor in an epithelial cell of the mesenteron to injure cells in the vicinity of it. In the injured part, the digestive juice and the body fluid mix with each other to change the osmotic pressure and pH in the body.

As a result, the food digesting function is disturbed, paralysis of mouth-part is caused, and the feeding action is retarded in the insect. Furthermore, the spores germinate under nutritional conditions and they invade into the hemocele of the insect as the vegetative cells propagate, thus causing blood poisoning.

Although insects may have different sensitivity depending on the species of insect, usually the feeding action ceases after several hours and the insect dies after 2 or 3 days after eating Bt. It is attributable to this phenomenon that less damage by insects' eating is observed even when some insects remain alive after Bt is used. Many synthetic insecticides act on the nerve system of insect so that vigorous convulsion or knockdown effect, paralysis or the like phenomenon is observed. However, the mechanism of the action of Bt is quite different as described above and the effect is gradually exhibited even though living insects exist after the treatment. Bt and protein having a pesticidal activity (crystalline toxic protein) produced by Bt are very useful as an environmentally safe microbial pesticides (Bt agents), in particular as insecticides for *Lepidoptera* insects and are practically used worldwide.

Bt is gram-positive rod cells and produces crystal protein in the spore formation stage at a late stage of logarithmic phase. The crystal protein is not converted into a protein having a pesticidal activity to cause gut paralysis and systemic paralysis before it is orally taken into the digestive tract by an insect to be subjected to alkali decomposition and enzymatic decomposition in the digestive juice. However, it does not exhibit toxicity to mammals.

The crystal proteins Bt produces are formed in the sporangium along with the spores and released to the outside of the cell together with the spores after passing the phase of the sporangium (Nature, 172, 1004, 1953). These generally constitute complex crystals such as diamond-shaped, bipyramidal, rhomboidal and so forth and are insoluble in water. They are produced one per spore at the time of spore formation and released together with spores into

medium as the bacterial cell is destructed. Usually they are of a steric rhombic or orthorhombic structure and have a size on the order of 2.0 μ in the long side and 0.6 μ in the short side. Subspecies include also amorphous ones and their size varies widely. On their surface, regular stripe structures can be seen. Isolation from the medium and purification of crystal protein can be performed by use of a bilayer fractionation method, a density gradient centrifugation method or the like.

The crystal proteins are soluble in an NaOH solution having a pH 12 or more. According to the analyses by SDS-PAGE (polyacrylamide gel electrophoresis), there are observed three proteins of about 130 to 135 kDa, about 65 kDa and about 80 kDa in a bacterial strain belonging to *Bacillus thuringiensis*. They are generically called Cry 1 protein, Cry 2 protein, and Cry 5 protein. Furthermore, they can be separated into a plurality of proteins that have almost approximate molecular weights but partially differ from each other by a fractionation operation such as high performance liquid chromatography. That is, in the case of Cry-1 protein, it is classified into proteins Cry-1Aa, Cry1Ab and so forth.

Bt was isolated from larva of Mediterranean flour moth (*Ephestia kuehniella* Zeller [Pyralidae]) by Berliner, a German researcher in 1911. Since the larva of the insect ate the flour from Thuringia, the insect was named *Thuringiensis*. Earlier than this, Dr. Ishiwatari isolated the same bacterial species as a pathogenic bacterium to silkworm in 1901. Thus, it is understood that Bt has widely occurred in the natural world since old. For example, it is present in grain warehouses and millhouses where grain pests inhabit. Also, it is detected in wagons and cabins and so forth for transporting grains. Thus it is known that it migrates everywhere in the world. Also in Japan, its distribution in every district has been examined and many *Bacillus thuringiensis* strains have been isolated from the dust in the houses of silkworm farmers, the surface of plants and so forth.

The bacteria that belong to the genus *Bacillus* amount to 70 or more species. Those strains frequently observed worldwide include 22 strains. They are distinguished basically by the ability of spore formation and shape of spore, production of gas, production of acetylmethylcarbinol (AMC), reduction of nitrates, and assimilability of some sugars in accordance with the techniques of Thiery and Franchon. *Bacillus thuringiensis* (*B. thuringiensis*) is finally distinguished from its allied species by the presence or absence of a crystal having a pesticidal activity ("Manual of techniques in insect pathology," L. Lacey ed., Academic Press, California, 55-77 (1997)).

The characteristics used for distinguishing *Bacillus thuringiensis* from other bacterial species and other species belonging to the genus *Bacillus* include gram-positive rod, catalase (+), spore formation (+), ovary spore, 0.9 μ or more in width of vegetative cell, production of acetylmethylcarbinol (+), facultative anaerobicity, assimilation of D-mannitol (-), and existence of crystal protein (+).

For the identification of subspecies of Bt, flagellum antigen (H-antigen) according to the serological technique by De Barjac and Bonefoi using an antibody in a rabbit serum to the flagellum of a bacterium has been employed for a long time as long as 40 years (Entomophaga 7, 5-31, 1962). This is a technique that has been widely utilized for the phylogenetic systematics of *Bacillus thuringiensis*.

The pesticidal activity of the bacterial strains varies depending on subspecies and is of an extremely high specificity. For example, there have been known *kurustaki*, *aizawai* and so forth as subspecies that exhibit an activity to *Lepidoptera* insects and *tenebrionis*, *japonensis* and so forth as subspecies that exhibit an activity to *Coleoptera* insects.

However, in actuality, bacterial strains belonging to the same subspecies may differ in the spectrum of pesticidal activity depending on the strain. In the case of Bt strains that have

an activity to a part of *Lepidoptera* insect pests, the pests have acquired a resistance thereto. In addition, few reports have been made on strains exhibiting effective activity to *Coleoptera* insects.

Thus, a novel Bt agent that is effective to *Lepidoptera* insect pests having acquired a resistance to the Bt agent is demanded. Furthermore, there is a keen demand for a Bt agent having an activity to *Coleoptera* insects. Among these, novel Bt agents having a pesticidal activity to larvae of *Coleoptera* insects, in particular larvae of scarabs, thus far reported include only *Bacillus thuringiensis Serovar, japonensis strain buibui*) strain (Japanese Patent Application Laid-open Nos. Hei 6-65292 and Hei 7-179) and *Bacillus thuringiensis var. japonensis* N141 (Japanese Patent Application Laid-open No. Hei 8-228783).

DISCLOSURE OF THE INVENTION

The conventional *buibui* strain or N141 strain belonging to the subspecies *japonensis* does not exhibit sufficient effect on larvae of scarabs, in particular larvae of *Anomala cuprea*, a serious pest for lawn grasses, taro, sweet potato, peanut and so forth, and *Anomala orientalis* and *Popillia japonica*, pests for lawn grasses. Furthermore, Bt toxins of the microbe that belongs to the same bacterial species (subspecies) exhibit crossing in case a resistance is developed in a part thereof, with the result that its effect is considerably decreased. On the other hand, Bt toxins take a certain time for their effect to be exhibited. Therefore, discovery of a novel toxin that has a more potent pesticidal activity is keenly desired.

Therefore, an object of the present invention is to provide a novel bacterial strain belonging to *Bacillus thuringiensis serovar galleriae* that produces a pesticidal protein

having a high pesticidal activity to larvae of *Coleoptera* insects and to provide a protein having a pesticidal activity derived from the novel microbe.

Furthermore, an object of the present invention is to provide a protein that has the above pesticidal activity, a protein that has an amino acid sequence obtained by addition, deletion or substitution of a plurality of amino acids in the amino acid sequence that constitutes the protein and has the same pesticidal activity, DNAs that encode such amino acid sequences, microbes that have been transformed by use of the DNAs and produce proteins having pesticidal activity, plants transformed by use of such DNAs or seeds thereof, and noxious organism-controlling agents and -controlling methods.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is an electron micrograph of *Bacillus thuringiensis serovar galleriae* SD502 strain.

Fig. 2 is a diagram illustrating the results of SDS-PAGE of crystal protein having a pesticidal activity of the present invention. 1 designates markers, showing 200, 116.25, 97.4, 66.2, and 45.0 kDa from the top. 2 represents the results of crySDS502 gene product expressed in *Escherichia coli*. 3 represents the results of SDS 502 crystal protein.

Fig. 3 is a diagram illustrating linkage of *Bacillus thuringiensis serovar galleriae* SD502 gene to a vector (gene cassette).

DETAILED DESCRIPTION OF THE INVENTION

With a view to finding out a novel microbe having a high effect on larvae of *Coleoptera* insects, the present inventors have conducted repeated analyses and as a result they have isolated a *Bacillus thuringiensis serovar galleriae* SD502 strain belonging to *Bacillus thuringiensis serovar galleriae* that produces a pesticidal protein having a high

pesticidal activity to larvae of *Coleoptera* insects. Thus, they have achieved the present invention that relates to a pesticide containing the novel *Bacillus thuringiensis serovar galleriae* SD502 itself and/or a pesticidal protein (toxic protein) it produces as active ingredient(s).

Furthermore, they have confirmed that a DNA encoding the pesticidal protein the novel microbe of the present invention produces, a protein having an amino acid sequence encoded by the DNA, and a noxious organism-controlling agent containing the protein as an active ingredient are effective as pest-controlling means and thus achieved the present invention.

That is, the present invention relates to (1) proteins having a pesticidal activity, (2) DNA encoding such proteins, (3) noxious organism-controlling agents, (4) plant protecting methods, (5) (5-1) microbes, (5-2) plants or seeds thereof transformed by use of the DNA, and (6) novel microbe, as set forth below.

A protein having an amino acid sequence described in SEQ.ID.NO:1 in the Sequence Listing and exhibiting a pesticidal activity.

A protein having an amino acid sequence derived by addition, deletion or substitution of a plurality of amino acids in the amino acid sequence described in SEQ.ID.NO:1 in the Sequence Listing and exhibiting a pesticidal activity.

A DNA containing a nucleotide sequence encoding the protein as described in 1) above.

The DNA as described in 3) above, containing a nucleotide sequence as described in SEQ.ID.NO:3 in the Sequence Listing.

A DNA containing a nucleotide sequence encoding the protein as described in 2) above.

A noxious organism-controlling agent, comprising

a microbe producing a protein having an amino acid sequence described in SEQ.ID.NO:1 in the Sequence Listing, selected from

(1-1) *Bacillus thuringiensis serovar galleriae* SD502 strain,

(1-2) a mutant thereof, and

(1-3) a microbe transformed with a DNA containing a nucleotide sequence encoding a protein having an amino acid sequence described in SEQ.ID.NO:1 in the Sequence Listing, or

a protein having a pesticidal activity, produced by a microbe selected from

(2-1) the above-mentioned SDS502 strain,

(2-2) its mutant, and

(2-3) transformed microbe.

A microbe transformed with the DNA as described in 5) above and producing a protein exhibiting a pesticidal activity as described in 2) above.

A plant transformed with the DNA as described in 3) or 5) above, or seed thereof.

A method for controlling a noxious organism, wherein the protein as described in 1) or 2) above is fed to a noxious organism to protect a plant from damage caused by the noxious organism.

The method for controlling a noxious organism as described in 9) above, wherein the noxious organism is a *Coleoptera* insect and the plant is protected from damage caused by the noxious organism.

Bacillus thuringiensis serovar galleriae SDS502 strain producing a protein having an amino acid sequence described in SEQ.ID.NO:1 in the Sequence Listing and exhibiting a pesticidal activity.

Novel *Bacillus thuringiensis serovar galleriae* SDS502 strain of the present invention has been internationally deposited at National Institute of Advanced Industrial Science and Technology, Independent Administrative Institution, under Accession No. FERM BP-7667.

The SDS502 strain can be cultured in a medium in which general bacteria can grow by a common fermentation technique.

Examples of medium include a common broth medium (0.3% of meat extract, 1.0% of peptone, and 0.5% of NaCl, pH 7.0), an MBS medium (0.7% of KH_2PO_4, 1% of bactotryptose, 0.2% of yeast extract, 0.03% of $MgSO_4 \cdot 7H_2O$, and 0.02% of $CaCl_2 \cdot 2H_2O$, pH 7.2), an MRVP medium (0.5% of polypeptone, 0.5% of glucose, and 0.5% of NaCl, pH 7.0) and so forth.

As the carbon source, glucose, fructose, saccharose, maltose, molasses, soluble starch, cornstarch and so forth may be utilized.

As the nitrogen source, ammonium chloride, ammonium sulfate, urea, yeast extract, peptone, soybean powder, casein and so forth may be utilized.

Furthermore, it is preferable that as other inorganic salts and vitamins, NaH_2PO_4, K_2HPO_4, $MnSO_4$, $FeSO_4$, $MgSO_4$, NaCl, molasses, yeast extract, EBIOS (vitamin preparation) and so forth be added. The pH is preferably 6 to 8. The incubation temperature is preferably 25 to 33°C. The incubation time is preferably 24 to 120 hours. The culture method is preferably the one under aerobic conditions, such as aerobic spinner culture.

In the case where pesticidal crystal protein is isolated from the culture broth after the incubation, a common centrifugal separation method, a filtration method and so forth may be utilized. Alternatively, the SDS502 strain and/or crystal protein the SDS502 strain produces may be used in the form of a mixture with vegetative cells and/or spores without separation therefrom.

Also, mutant strains that produce a pesticidal crystal protein may be obtained from the SDS502 strain as an original strain by spontaneous or induced mutation, which strains may be used as an insecticidal crystal protein-producing strain according to the present invention. As the method for making mutant strains, a common method conventionally known can be used, for example, a method in which an original strain is subjected to artificial mutation by irradiation of ultraviolet rays or with a chemical such as N-methyl-N'-nitro-N-nitrosoguanidine (NTG), spread on an agar medium containing skimmed milk, screening a colony forming a greater clear zone around the colony from among the strains that grow thereon, and screening a strain having an excellent productivity.

In the case where a noxious organism-controlling agent containing the SDS502 strain and/or SDS502 crystal protein as an active ingredient or ingredients, it may be made into an optional formulation such as wettable powder, granules, dust, flowable formulation in the same manner as in common pesticides. These are used in admixture with a suitable carrier for respective formulations, for example, powder of a mineral such as agalmatolite, talc, kaolin, calcium carbonate, bentonite, silica stone powder, limestone powder, acid clay, diatomaceous earth powder, gypsum, pumice powder, shell powder, mica powder, or colloidal hydrated sodium silicate, water, or aqueous solutions such as buffer solutions. Preferably, they are used after addition of a fixing agent such as an alkylbenzenesulfonate or an alkylsulfonate, a humectant such as a polyoxyethylene (POE) alkyl ether, a POE alkyl phenyl ether, a POE dialkyl phenyl ether, a POE alkylamine or a dialkyl sulfosuccinate, a dispersing agent such as an alkyl sulfate, a POE alkyl ether sulfate, a POE alkyl phenyl ether sulfate, a POE benzylated (or salicylated) phenyl (or phenylphenyl) ether sulfate, a paraffin (alkane) sulfonate, an alpha-olefin sulfonate (AOS), an alkyl benzenesulfonate, a mono- or dialkyl naphthalene sulfonate, a naphthalenesulfonate/formaldehyde condensate, an alkyl

diphenyl ether disulfonate, a lignin sulfonate, a POE alkyl ether sufosuccinate half ester, or a POE benzyl (or styrylated) phenyl (or phenylphenyl) ether phosphate, a mildewproofing agent such as a paraoxybenzoic acid derivative, salicyl anilide, 1,2-benzoisothiazolin-3-one, tetraphthalonitrile (TPN) or 2-nitrobromo compound.

In contrast to using the SDS502 strain and/or SDS502 strain-produced crystal protein as a single active ingredient, it is also possible to mix it with herbicides, various pesticides, bactericides or plant growth regulators which are effective to other noxious organisms, synergists for multiplying the effect, attractants as well as plant nutritive agents, fertilizers and so forth that are intended to obtain other functions.

In preparing a noxious organism-controlling agent containing the SDS502 strain and/or SDS502 strain-produced crystal protein as an active ingredient or ingredients, its active ingredient content is suitably on the order of 10 to 99%, preferably 40 to 90%. However, the active ingredient content may be adjusted depending on the target noxious organism, cultivated crop, method of use, time of use and so forth.

The crystal protein of the present invention includes, in addition to those having the amino acid sequence as described in SEQ.ID.NO:1 in the Sequence Listing, also those having the one that is partly deficient (for example, a polypeptide composed of only a portion that is necessary for the expression of bioactivity out of the amino acid sequence as described in SEQ.ID.NO:1 in the Sequence Listing), those having the one partly substituted with other amino acids (for example, the one substituted by amino acids having similar physical properties), and those in which other amino acids are added or inserted in some part thereof.

As is well known in the art, 1 to 6 kinds of codon are known to code for one amino acid (for example, one kind for Met and 6 kinds for Leu). Therefore, the nucleotide sequence of DNA can be altered without altering the amino acid sequence of a polypeptide.

Examples of the pests that can be controlled by the method of the present invention include the following *Coleoptera* insects. That is, scarabs such as *Anomala cuprea*, *Anomala diversa*, *Anomala octiescostata*, *Hoplia communis*, *Ectinohoplia obducta*, *Anomala orientalis*, *Anomala osakana*, *Anomala testaceipes*, *Anomala schonfeldti*, *Anomala rufocuprea*, *Anomala albopilosa*, *Maladera castanea*, *Melolontha japonica*, *Adoretus tenuimaculatus* and *Popillia japonica*, ladybugs such as *Epilachna vigintioctopunctatav* and *Epilachna vigintioctomaculata*, weevils such as *Lissorhoptrus oryzophilus*, *Scepticus griseus*, *Cylas formicarius*, *Sphenophrus venatus vestius* and *Sitophilus zeamaise*, leaf beetles such as *Phyllotreta striolata* and *Aulacophora femoralis*, click beetle such as *Melanotus okinawaensis*, long-horned beetles such as *Monochamus alternatus* and *Mesosa myops*, bark beetles such as *Scolytus japonicus* and *Xylosandrus germanus*, and confused flour beetles such as *Tenebrio molitor* and *Tribolium castaneum*.

The method for controlling a noxious organism of the present invention using a noxious organism-controlling agent that contains the SDS502 strain and/or SDS502 crystal protein as an active ingredient or ingredients can be used for protecting a wide variety of plants that are susceptible to attack of *Coleoptera* insect pests. Specific examples of target plant include vegetables such as Chinese cabbage and cabbage, fruit vegetables such as cauliflower, root crop such as sweet potato or taro, citrus, defoliating fruit trees, cereals such as rice, wheat and beans, lawn grass in golf courses, gardens and so forth, specialty crop such as tea or sugarcane, stored cereals, stored food and flower trees. Also, the method of the present invention can be used for trees in forestation and non-agricultural areas such as parks, trees in forests, and seedling and so forth.

Generally, the method for protecting plants from insect damages by *Coleoptera* insect pests by use of a noxious organism-controlling agent that contains the SDS502 strain and/or

SDS502 crystal protein as an active ingredient or ingredients can be practiced by treating (for example, spraying on) a plant where insect pests proliferate or will tend to proliferate with a composition of the above-mentioned noxious organism-controlling agent diluted with a diluent such as water, or directly mixing or injecting into soil without dilution.

The SDS502 gene can be isolated from the SDS502 strain. Total DNA of the SDS502 strain is digested with one or more restriction enzymes and the produced DNA fragment is converted to a 2- to 5-kbp DNA fraction. The fraction is linked to a suitable vector and *Escherichia coli* is transformed therewith. Next, using an antibody to the pesticidal crystal protein the SDS502 strain produces, an enzyme immunoassay method is practiced and thus an *Escherichia coli* transformant having the objective gene can be obtained.

The crystal protein gene DNA derived from the SDS502 strain thus obtained is treated with a suitable restriction enzyme or enzymes and the obtained DNA fragment is coupled to a suitable cloning vector to make a gene cassette. Using this, microbes such as *Escherichia coli* and *Bacillus subtilis* can be transformed. For example, *Escherichia coli* can be transformed and a nucleotide sequence encoding the SDS502 strain produced crystal protein can be analyzed by a gene analysis method such as a dideoxy method.

Using the gene cassette, gram-positive bacteria having a pesticidal activity, for example, *Bacillus thuringiensis serovar galleriae* or other subspecies can be transformed. This enables one to obtain transformed *Bacillus thuringiensis* effective for controlling a broader range of insects.

Furthermore, to express the SDS502 gene in plants, a preferred restriction site may be introduced so as to be located on a flank of each gene or gene part to induce mutation of a specified site.

The SDS502 gene part encoding the active part of the pesticidal crystal protein of the SDS502 strain can be stably inserted in the nuclear genome in a single plant cell to make a transformed plant having a resistance to insects or having the ability of killing insects.

As a result, using the obtained transformed plant, transformed plants having the same characteristics can be produced. Furthermore, the SDS502 gene part having a resistance to insects or the ability of killing insects can be introduced into other variants of the same or related plant species. The seeds obtained from the transformed plants are stable genome inserted products that contain the SDS502 gene part that can exhibit a resistance to insects or insecticidal activity and that is effective as a pesticide.

The SDS502 strain may be further transformed with one or more exogenous Bt genes having pesticidal activities. For example, the noxious organisms on which the SDS502 strain and/or SDS502 strain-produced crystal protein has no activity includes in particular larvae of *Lepidoptera* insects. Now, a chimera gene of the SDS502 gene with a gene encoding a crystal protein derived from other microbe exhibiting an effective activity on them may be prepared and used for transforming the microbe to the one having a wider pesticidal spectrum. By so doing, transformed SDS502 strains that can control a wider variety of pests are produced.

Antibody specific to SDS502 strain crystal protein can be prepared by immunizing a guinea pig with the crystal protein of the SDS502.

BEST MODE FOR CARRYING OUT THE INVENTION

Hereinafter, the present invention will be illustrated by way of Examples. However, the present invention is by no means limited by the following Examples.

Example 1: Isolation of *Bacillus thuringiensis serovar galleriae* SDS502 Strain

From soil collected in the city of Tsukuba, a *Bacillus thuringiensis serovar galleriae* SDS502 strain was isolated by use of the following technique.

10 mg of sample soil was charged in an Erlenmeyer flask and 10 ml of sterilized water was poured therein. After shaking for 30 minutes, the mixture was left to stand for a while. Then, 2 ml of supernatant was taken out and immediately heated at 80°C for 10 minutes. The heated liquid was diluted in two stages to 10 folds and further to 100 folds. 1 ml each of dilutions was incubated on an NB plate medium (0.3% of meat extract, 1.0% of peptone, 10.5% of NaCl, 2% of agar, pH 7.0/distilled water) at 30°C for 24 to 48 hours.

Out of the obtained colonies, white, rough-edged and rapidly growing colonies were selected to obtain *Bacillus thuringiensis* in a high probability.

Example 2: Bacteriological Properties of *Bacillus thuringiensis serovar galleriae* SDS502 Strain

Method: Search was conducted in accordance with the taxonomy and bacteriological techniques described in Cowan. S. T., "Manual of Identification of Medical Bacteria" (translated by T. Sakazaki, Kindai Shuppan).

Gram stain: Gram-positive rod,

Morphology of colony: Forms an opaque beige colony having irregular edges,

Spore forming ability and shape of spore: (+) oval spore;

Catalase: (+),

Width of vegetative cell: 0.9μ or more,

Production of AMC: (+),

Respiration: Facultative an aerobic,

Assimilation of D-Mannitol: (-),

Existence of crystal protein: (+),

Serum type of flagella: H anti-serum type (5a5b),

Cell contents: Spore forming cells produce amorphous type crystal protein (cf. Fig. 1),

Alkali-soluble protein: (+) protein electrophoresed near 130 kDa (cf. Fig. 2),

Activity: The strain of the invention has lethal activity on *Coleoptera* pests tested.

From the above findings, the strain of the invention was judged to be a novel strain. This was named *Bacillus thuringiensis serovar galleriae* SDS502 and deposited at Laboratory of Microbial Industry and Technology, Institute of Industrial Science Technology, Ministry of International Trade and Industry, (now National Institute of Advanced Industrial Science and Technology, Independent Administrative Institution) under Accession No. FERM P-17979 and transferred to International Deposition under International Receipt No. FERM BP-7667.

Example 3: Identification of Subtype of *Bacillus thuringiensis serovar galleriae* SDS502 strain

By use of an antibody to a protein of the flagellum of a serotyping *Bacillus* prepared with an antibody derived from an antigen of flagellum, an antigen-antibody reaction was carried out using a flagellum protein of an unknown bacterium as an antigen.

Flagellum H serum was prepared by heating the bacteria cells at 100°C to peel flagella off. Using 40 kinds (subtypes) of H antigen standard strains of *Bacillus thuringiensis* that are already known, bacteria having good mobility were selected using a Craigie tube (0.5% semi-fluid agar medium) and formalized dead bacteria were prepared from them. The formalized dead bacteria were given to a rabbit to immunize it. H serum was prepared by absorbing a corresponding antibody to the *Bacillus thuringiensis* cell antigen from each antiserum. The

serum type of H antigen and agglutinin value of the antibody were identified and quantitatively determined according to the method of Ooba and Ayusawa (I. Invertebr. Pathol., 32, 303-309, 1978).

The H antigen to *Bacillus thuringiensis serovar galleriae* SDS502 strain specifically agglutinates *serovar galleriae* only. The agglutinin value of *serovar galleriae* SDS502 strain H antiserum to a corresponding homo antigen was 12,800 folds and the agglutinin value of it to *serovar galleriae* HD8 strain (standard strain) was 6,400 folds. Therefore, SDS502 strain and *serovar galleriae* were judged to be the same strain.

Example 4: Purification of Crystal Protein of SDS502 Strain and Properties Thereof

One platinum loop of SDS502 strain cells were taken out and inoculated in a test tube containing common bouillon medium (0.3% of meat extract, 1.0% of peptone, 0.5% of NaCl, pH 7.0/distilled water). Reciprocating shaking culture of it was performed at 30°C for 24 hours to obtain a seed culture solution. The seed culture was inoculated in a 500 ml Erlenmeyer flask containing 100 ml of the above-mentioned medium such that the seed culture was in a final concentration of 1% and rotation shaking culture was performed at 30°C for 96 hours at 250 rpm. Then, cells, spores and crystal protein were recovered by centrifugation. A suitable amount of buffer (Tris-HCl, NaCl, EDTA) was added to the obtained precipitate and supersonic destruction was performed to obtain a suspension. The obtained suspension was subjected to 8% SDS-PAGE gel electrophoresis to examine its electrophoretic pattern. Also, using an antibody, Western blotting was performed. As result, it was confirmed that there existed a crystal protein having molecular weight of about 130 kDa produced by the SDS502 strain.

Example 5: Pesticidal Activity of SDS502 Strain on *Anomala cuprea*, *Popillia japonica*, *Anomala orientalis*, *Plutella xylostella* and *Bombyx mori*.

The suspension prepared in Example 4 was diluted to a crystal protein concentration of 10 μg/ml and a spreading agent was added thereto to obtain a sample solution. The sample solution was mixed with leaf mold that had been subjected to sterilization treatment in advance and 1st stage, 2nd stage and 3rd stage larvae of *Anomala cuprea*, 1st stage and 2nd stage larvae of *Popillia japonica*, as well as 1st stage and 2nd stage larvae of *Anomala orientalis* were released.

Furthermore, the leaf of cabbage was dipped in the sample solution and thereafter it was sufficiently air-dried. This was placed in a Styrol cup containing wet filter paper. In the cup, larvae of *Plutella xylostella* in the middle phase of 3rd stage were released. After 7 days (after 5 days in the case of *Bombyx mori*, or after 2 days in the case of *Plutella xylostella*), the mortality of larvae was obtained according to the following formula. The tests were performed in 5 series with 5 insects per lot.

Mortality (%) = (Number of dead insect/Number of released insect) x 100

Furthermore, the sample solution mixed with 5 g of artificial feed stuff was charged in a dish. In the dish, larvae of *Bombyx mori* on the 2nd day in the 3rd stage were released and the mortality of larvae after 7 days (after 5 days in the case of *Bombyx mori*, or after 2 days in the case of *Plutella xylostella*) was obtained according to the above-mentioned formula. The tests were performed in 5 series with 5 insects per lot. As a control, a test solution of the pesticidal protein produced by *Bacillus thuringiensis serovar galleriae* HD8 strain (standard strain) was prepared in the same manner as above and comparison therewith was made.

As a result, as indicated in the pesticidal spectrum of the crystal protein produced by *Bacillus thuringiensis serovar galleriae* SDS502 strain (Table 1), the pesticidal protein

produced by the SDS502 strain exhibited pesticidal effect to *Anomala cuprea Hope*, *Anomala orientalis*, and *Popillia japonica* belonging to *Coleoptera* in a concentration of 10 μg/ml while the crystal protein produced by *Bacillus thuringiensis serovar galleriae* HD8 strain (standard strain) exhibited no pesticidal effect. On the other hand, the HD8 strain (standard strain) exhibited high activity to larvae of *Bombyx mori*, *Plutella xylostella*, and *Spodoptera litura* belonging to *Lepidoptera* while the SDS502 strain exhibited no activity to *Lepidoptera* insects except for *Plutella xylostella*. These results suggest that the crystal proteins have different compositions and the strains cannot be said to be completely the same strain in consideration of the facts that the *galleriae* standard strain has cry1Ab gene and exhibits a pesticidal effect to *Coleoptera* while the SDS502 strain exhibits substantially no pesticidal activity to *Lepidoptera*.

Table 1

Mortality (%) After 7 Days From Eating Crystal Protein 10 μg)

Name of Insect	*Bacillus thuringiensis serovar galleriae*	
	SDS 502 Strain	HD8 Strain (Standard Strain)
Anomala cuprea larvae (1st stage Larvae)	100	0
Anomala cuprea larvae (2nd stage Larvae)	100	0
Anomala cuprea larvae (3rd stage Larvae)	80	0
Popillia japonica (1st stage Larvae)	100	0
Popillia japonica (2nd stage Larvae)	100	0
Anomala orientalis larvae (1st stage Larvae)	100	0
Anomala orientalis larvae (2nd stage Larvae)	100	0
Bombyx mori *	0	80
Plutella xylostella **	40	80
Spodoptera litura	0	40

* Examiner after 5 days; **Examiner after 2 days

Example 6: Gene Relating to Pesticidal Protein of *Bacillus thuringiensis serovar galleriae* SDS502 Strain

An antibody obtained by immunizing a guinea pig with about 130 kDa crystal protein produced by *Bacillus thuringiensis serovar galleriae* SDS502 strain was used for cloning a gene encoding SDS502 strain crystal protein (hereinafter, abbreviated as SDS502 gene). The cloned gene had 3,690 nucleotides and contained a translation region ranging from 187th ATG codon to 3,688th TAA codon. Further, it was compared with known genes, i.e., *japonensis buibui* gene (Japanese Patent Application Laid-open No. H6-65292) and *japonensis* N141 gene (Japanese Patent Application Laid-open No. H8-228783) effective to

Coleoptera insects. As a result, it revealed that the both genes showed only 71% and 42% homologies, respectively, in amino acid sequence.

Example 7: Isolation and Cloning of SDS502 Gene

Total DNA was prepared from SDS502 strain and partially cleaved with restriction enzyme EcoRI. From the cleaved DNA, about 2 to 5 kbp DNA fragment was obtained by fractionation and ligated with a phage vector (λgt11) cleaved with EcoRI. *Escherichia coli* was transformed with the product. Then, the recombinant *Escherichia coli* clone was subjected to antibody screening with an antibody obtained by immunizing a guinea pig with the about 130-kDa protein that was considered to be SDS502 stain crystal protein to identify a clone containing the SDS502 gene. DNA was prepared from the recombinant *Escherichia coli* clone and the DNA was cleaved with restriction enzyme EcoRI. The cleaved DNA fragments were electrophoresed on 0.8% agarose gel to identify an about 3.4-kbp insert DNA fragment.

The obtained DNA fragment was fractionated and ligated to Bluescript II SK(-), which was a plasmid vector cleaved with EcoRI, to prepare a gene cassette (pSDS502) (Fig. 3). Since this pSDS502 was not of full length, cloning was performed again to obtain a full-length pSDS502. Thereafter, the nucleotide sequence of the DNA fragment containing full-length SDS502 gene was determined by dideoxy method.

Example 8: Expression SDS502 Crystal Protein in *Escherichia coli* (*E. coli*:DH5α) and Properties of Expressed Protein

To produce crystal protein using the SDS502 gene, *Escherichia coli* (*E. coli*: DH5α) was transformed by use of the gene cassette (pSDS502) to obtain a recombinant *Escherichia*

coli (hereinafter referred to as *E. coli*:DH5α(pSDS502)). The recombinant *Escherichia coli* was incubated in LB-amp liquid medium (10 g of Trypton, 10 g of NaCl, 5 g of yeast extract, 0.2% of glucose, 50 mg/l of sterilized water of ampicillin) at 37°C for about 3 hours. Then, isopropyl 1-thio-β-D-galactoside (IPTG) was added thereto to a final concentration of 1 mM and the cultivation was continued for additional 20 hours at 37°C. After completion of the cultivation, the culture was centrifuged. Lysisbuffer was added to the precipitate in an amount of 4 folds (W/V) and the mixture was suspended at room temperature for 10 minutes. Then Lysozyme was added thereto to a final concentration of 1 mg/ml and after mixing, the mixture was left to stand on ice for 10 minutes. Further, Triton X-100 was added to this to a final concentration of 1% and after mixing, the mixture was centrifuged and the supernatant portion thereof was recovered. The obtained supernatant was subjected to 8% SDS-PAGE gel electrophoresis to examine its electrophoretic pattern. Also, Western blotting by use of an antibody was performed. As a result, it was observed that *E. coli*:DH5α(pSDS502) produced a crySDS502 crystal protein.

Example 9: Pesticidal Activity of Crystal Protein Derived from *E. coli*:DH5α(pSDS502) on 1st stage larvae of *Anomala cuprea* and of *Popillia japonica*

The supernatant solution obtained as described above was diluted such that the concentration of crystal protein was 10 μg/ml and a spreading agent was added thereto to make a sample solution. The sample solution was mixed with leaf mold that had been subjected to sterilization treatment in advance and 1st stage larvae of *Anomala cuprea* and of *Popillia japonica*, were released. As a result, it was confirmed that the sample solution exhibited pesticidal activity on *Anomala cuprea* and *Popillia japonica*.

INDUSTRIAL APPLICABILITY

According to the present invention, a novel microbe *Bacillus thuringiensis serovar galleriae* SDS502 strain having ability of producing a toxic protein having high pesticidal activity on *Coleoptera* larvae has been found out and also a gene encoding the pesticidal crystal protein and pesticidal crystal protein have been found out. Furthermore, by formulating a noxious organism-controlling agent comprising the protein as an active ingredient, a noxious organism-controlling agent having activity on noxious organisms that have acquired a resistance to the conventional Bt can be provided. In particular, the noxious organism-controlling agent of the present invention is superior in effect on larvae of *Anomala cuprea*, which is a strong pest for lawn grasses, taro, sweet potato, peanut and so forth, and on *Anomala orientalis* and *Popillia japonica* and so forth, which are pests for lawn grasses, with better cost performance, as compared with the conventional pesticides produced by chemical synthesis and the *buibui* strain belonging to the subgenus *japonensis*.